Biological Forum – An International Journal **14(2): 1206-1210(2022)**

ISSN No. (Print): 0975-1130
ISSN No. (Online): 2249-3239

Efficacy of New Generation and Combination Fungicides under *in vitro* for the Management of Anthracnose of Nutmeg (*Myristica fragrans* Houtt.)

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(Received 02 April 2022, Accepted 01 June, 2022)
(Published by Research Trend, Website: www.researchtrend.net)

ABSTRACT: Anthracnose and fruit rot caused by *Colletotrichum gloeosporioides* is one of the major fungal diseases drastically reducing the yields of nutmeg. The information on practical application of new generation fungicides *i.e,* triazoles, strobilurins and combination fungicides in the perennial tree spice crops is limited and thus there is a need for developing a new management strategy with new generation fungicides. In this background, during 2019, a comprehensive and systematic survey was carried out in four districts of Kerala, namely Thiruvananthapuram, Kottayam, Ernakulam, and Idukki, to collect anthracnose-infected nutmeg samples. In the surveyed area, anthracnose symptoms ranged from necrotic spots with a prominent yellow halo to leaf blight or shot hole to fruit rot. Eighteen isolates of *Colletotrichum* sp. were obtained from anthracnose infected nutmeg and confirmed all the isolates as *C. gloeosporioides*. In the present study, the most virulent isolate is tested for *in vitro* efficacy of new generation (Propiconazole 25 EC, Difenconazole 25 EC, and Azoxystrobin 23 SC) and combination fungicides (Carbendazim 12% + Mancozeb 63 % WP and Trifloxystrobin 25% + Tebuconazole 55 % WP) at four different concentrations, namely 10, 25, 50, and 100 ppm, using the poisoned food technique. *In vitro* testing of new generation fungicides revealed that Propiconazole 25EC at 100 ppm and combination fungicides, Carbendazim 12 percent + Mancozeb 63 percent WP at 25 ppm; and Trifloxystrobin 25 percent + Tebuconazole 55 percent WP at 100 ppm, resulted in 100% inhibition of the mycelial growth of the pathogen. At 100 ppm, Difenoconazole 25 EC, Azoxystrobin 23 SC, and Captan 50 percent WP + Hexaconazole 5 percent WP inhibited mycelial growth by 69.33, 73.33, and 79.10 percent, respectively. Mycelial growth in fungicide-affected medium was either fluffy or cottony. Among the fungicides tested, Carbendazim 12% + Mancozeb 63 % WP inhibited pathogen mycelial growth even at low concentrations of 25 ppm, 50 ppm and 100 ppm with 100% efficacy indicating a promising direction for disease management while other fungicides were found least effective.

Keywords: Anthracnose, *Colletotrichum*, Fungicides, Management, Nutmeg.

INTRODUCTION

Nutmeg (*Myristica fragrans* Houtt.) is a perennial evergreen tree spice native to Indonesia's Moluccas islands and a member of the Myristicacea family. It is known as a twin spice because it produces two products, nutmeg from seed and mace from seed covering, both of which are used as spices due to their flavour, aroma, and fragrance. When used in small amounts, it has medicinal properties that aid in the relief of diarrhoea, vomiting, dizziness, and other symptoms. Nutmeg has a high market value for value-added products such as nutmeg syrup, nutmeg jelly, and nutmeg candy. India is the largest exporter of nutmeg, in which Kerala, Karnataka, Tamil Nadu, Goa and konkan are the leading producers. The production of nutmeg in India during 2016–17 was about 16,000 MT from an area of 23,000 ha (GOI, 2017). Kerala contributes to a production of 13,746 tons from an area of 22,065 ha (GOK, 2017).

Various diseases, including leaf spot, thread blight, fruit rot, dieback, and twig blight, have an impact on crop productivity. Anthracnose, caused by *Colletotrichum gloeosporioides*, is one of the principal pathogens that causes significant economic loss due to fruit rot. It was first reported from Kerala in 1961 as an unknown species of *Colletotrichum* later confirmed as *C. gloeosporioides* by Nair *et al.* (1978). Current management strategy of anthracnose disease includes spraying of 1% Bordeaux mixture, biocontrol practices of applying *Pseudomonas flourescens*, *Trichoderma* etc. in which the complete management is not achieved. Considering the limitations in the practical usage of new generation fungicides *i.e*, Triazoles, Strobilurins and combination fungicides in the perennial tree spice crops requires a need for developing a new management strategy with new generation fungicides. Accordingly, to the above facts and research gaps, the present study was conducted with the main objective to

identify the best treatment in the management of the anthracnose of nutmeg.

MATERIALS AND METHODS

Isolation of the pathogen, purification and maintenance of culture. The infected samples collected from different nutmeg growing areas were isolated using the standard procedure for tissue isolation. The isolated cultures were purified by single spore technique (Dhingra and Sinclair 1993). The purified cultures were maintained on Potato dextrose agar (PDA) slants. The pathogenicity was proved by artificial inoculation of *Colletotrichum* sp. into the healthy nutmeg leaves.

Screening of fungicides. The *C. gloeosporioides* isolate was tested with different commercially available new generation fungicides *i.e.* two triazoles (propiconazole and difenoconazole), one strobilurin (azoxystrobin,) and three combination fungicides (carbendazim + mancozeb, trifloxystrobin + tebuconazole, captan + hexaconazole) by using poisoned food technique (Nene and Thapliyal 1993) at four different concentrations (10, 25, 50 and 100 ppm) (Table 1).

Table 1: Different fungicides used in the *in vitro* efficacy.

Sr. No.	Fungicide		Formulation
	Chemical name	Trade Name	
1.	Propiconazole	Tilt	25 EC
2.	Difenoconazole	Score	25 EC
3.	Azoxystrobin	Amistar	23 SC
4.	Captan 50% WP + Hexaconazole 5% WP	Taaqat	50% WP + 5% WP
5.	Trifloxystrobin 25% + Tebuconazole 55% WP	Nativo	25% + 55% WP
6.	Carbendazim 12% WP + Mancozeb 63% WP	Saaf	12% WP + 63% WP

C. gloeosporioides isolate was allowed to grow in the sterile petri dishes containing PDA medium for seven days. Double strength PDA medium of 50 ml and 50 ml of sterile water were prepared for the respective fungicides of different concentrations and autoclaved. Under aseptic conditions, pre-weighed fungicides of different concentrations were dispersed into the conical flasks containing 50 ml sterile water and shaken thoroughly for the complete dispersion of the fungicide. In case of soluble concentrate fungicides, the desired concentrations were taken out by using the micropipettes and allowed to dissolve in the sterile water by thorough shaking. Melted medium was mixed with sterile water containing fungicide. The amended molten medium was poured into the sterile Petri dishes and allowed to solidify. This method was repeated for the different concentrations of each treatment.

After the solidification of the medium, mycelial discs of 5 mm diameter were cut by using the sterile cork-borer from the seven-day old culture plate. The mycelial discs were taken by flame sterilized inoculation needle and placed on the centre of the solidified PDA medium amended with the fungicide. Three replications for each concentration of different fungicides were maintained. A control plate inoculated with the pathogen alone and without the fungicide was maintained as control. The Petri dishes were then wrapped and incubated at the room temperature of 28$\pm$3°C. The radial growth of the pathogen was recorded when the pathogen in the control plate was fully grown and the percent inhibition by the fungicide was calculated. Percent inhibition of the pathogen by the fungicide over the control was calculated by the formula:

$$\text{Percent inhibition} = \text{C-T/C} \times 100$$

Where C= Radial growth of the pathogen in control plate in cm

T= Radial growth of the pathogen in the treatment plate in cm

RESULTS AND DISCUSSIONS

In vitro screening was carried out to find out the most effective fungicide against *C. gloeosporioides* by poisoned food technique. Different fungicides used in the experiment were Propiconazole 25 EC, Difenoconazole 25 EC, Azoxystrobin 23 SC, Captan 50% WP + Hexaconazole, 5% WP, Trifloxystrobin 25% + Tebuconazole 55% WP and Carbendazim 12% WP + Mancozeb 63% WP. These fungicides were evaluated at four different concentrations of 10, 25, 50 and 100 ppm. Present study of *in vitro* screening of new generation fungicides revealed that triazole fungicide Propiconazole 25EC at 100 ppm and combination fungicides, Carbendazim 12% + Mancozeb 63% WP at 25 ppm and Trifloxystrobin 25% + Tebuconazole 55% WP at 100 ppm concentration resulted in cent per cent inhibition of the mycelial growth of the pathogen. Difenoconazole 25 EC, Azoxystrobin 23 SC and Captan 50% WP + Hexaconazole 5% WP showed mycelial inhibition of 69.33, 73.33 and 79.10 per cent respectively at 100 ppm. (Table 3 and Fig. 1 and 2). Ashoka (2005) reported Carbendazim + mancozeb and benomyl showed cent per cent mycelial inhibition of *C. gloeosporioides* @ 0.025%, 0.005% and 0.1% from vanilla. Chandrakrant (2005) reported the triazole fungicides, propiconazole, difenoconazole and hexaconazole at 0.05 per cent had completely inhibited mycelial growth of *C. gloeosporioides* from black pepper. Similar results were reported by Jadhav *et al*. (2008) where Carbendazim + mancozeb @ 0.25 per cent and propiconazole @ 0.1 per cent showed cent per cent inhibition of mycelial growth of *C. gloeosporioides*. However, the nature of mycelial growth of *C. gloeosporioides* (C4) in fungicide amended PDA medium presented and summarized in Table 2.

Table 2: Nature of mycelial growth of *C. gloeosporioides* (C4) in fungicide amended PDA medium (Poisoned food technique).

Treatments	Dosage	Nature of mycelial growth	
		Front view	Rear view
T1-T4: Propiconazole 25 EC	10 ppm	White fluffy growth with regular margin	Orange colour
	25 ppm	Whitish fluffy growth with regular margin	Orange with grey radial zones
	50 ppm	Fluffy growth	Orange colour
	100 ppm	No growth	No growth
T5- T8: Difenoconazole 25 EC	10 ppm	White cottony growth	Orange colour with dark centre
	25 ppm	White cottony growth	Grey centre and white margin
	50 ppm	White cottony growth	Grey centre and white margin
	100 ppm	White cottony growth	Grey centre and white margin
T9-T12: Azoxystrobin 23 SC	10 ppm	White cottony growth	White with orange centre
	25ppm	White cottony growth	White with orange centre
	50 ppm	White cottony growth	White with orange centre
	100 ppm	White cottony growth	White with orange centre
T13– T16: Captan 50% WP + Hexaconazole 5% WP	10 ppm	White cottony growth with grey centre	Off white with orange centre
	25 ppm	Grey cottony growth	Off white with orange centre
	50 ppm	White cottony growth	Off white with orange centre
	100 ppm	Off white cottony	White with grey centre
T17- T20: Trifloxystrobin 25% + Tebuconazole 55% WP	10 ppm	White fluffy growth	White with grey centre
	25 ppm	White fluffy growth	White with grey centre
	50 ppm	White fluffy growth	White with grey centre
	100 ppm	White fluffy growth	White
T21 -T24: Carbendazim 12% WP + Mancozeb 63% WP	10 ppm	White cottony growth	White with orange centre
	25 ppm	-	-
	50 ppm	-	-
	100 ppm	-	-
T25: Control		White fluffy mycelium	White with grey radiating zones

Table 3: Percentage mycelial inhibition of *C. gloeosporioides* by new generation fungicides.

Treatments (Fungicides)		Percent inhibition (%) at different concentrations* (7 DAI)			
		10 ppm	25 ppm	50 ppm	100 ppm
T1- T4	Propiconazole 25 EC	60.88 (51.28)[a]	80.22 (63.57)[b]	85.55 (70.11)[b]	100.00 (90.00)[a]
T5-T8	Difenoconazole 25 EC	53.33 (46.90)[b]	56.21 (48.57)[c]	60.88 (51.28)[c]	69.33 (56.36)[c]
T9-T12	Azoxystrobin 23 SC	57.10 (49.08)[b]	56.88 (48.94)[c]	64.88 (53.64)[c]	73.33 (58.89)[c]
T13-T16	Captan 50% WP + Hexaconazole 5% WP	39.10 (38.52)[c]	48.21 (43.95)[c]	57.55 (49.34)[c]	79.108 (68.29)[b]
T17-T20	Trifloxystrobin 25% + Tebuconazole 55% WP	76.21 (60.80)[a]	79.32 (62.95)[b]	83.99 (66.42)[b]	100.00 (90.00)[a]
T21-T24	Carbendazim 12% WP + Mancozeb 63% WP	44.44 (41.65)[b]	100.00 (90.00)[a]	100.00 (90.00)[a]	100.00 (90.00)[a]
CD (0.05)		5.927	2.602	6.892	10.685
SEm		2.019	0.886	2.347	3.639

*Mean of five replication; DAI – days after inoculation; Values in parenthesis are arc sine transformed values

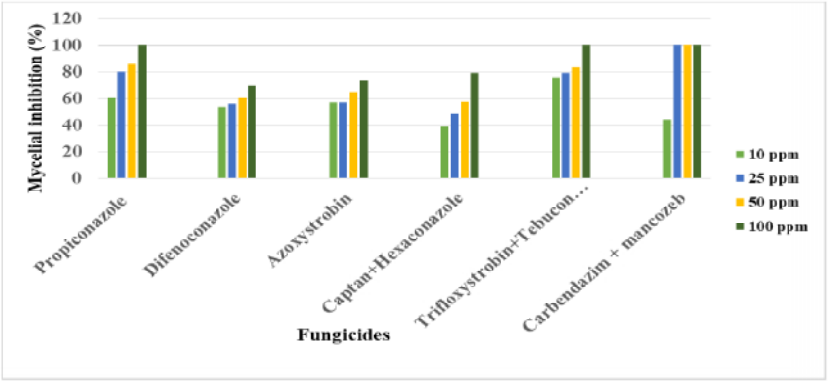

Fig. 1. Mycelial growth inhibition of C4 isolate at different concentration of fungicides on PDA medium at 7 DAI.

Positive correlation of result of the present study was reported from the *in vitro* studies conducted by Kurian *et al*. (2008). They reported effective control of black pepper anthracnose isolate of *C. gloeosporioides* using carbendazim + mancozeb @ 0.1 per cent. Prashanth *et al*. (2008) also reported pomegranate anthracnose caused by *C. gloeosporioides* was effectively inhibited by carbendazim + mancozeb @ 0.2 per cent (100 per cent inhibition). Carbendazim + mancozeb (89.23%); and propiconazole and difenoconazole (90.78%) were also effective at 0.1 per cent concentration. The triazole fungicides propiconazole, difenoconazole and

hexaconazole (0.1%) completely inhibited mycelial growth of *C. gloeosporioides* from sapota (Patil *et al*., 2010). Propiconazole and tebuconazole at 400 ppm effectively inhibited mycelial growth of *C. gloeosporioides* of mango (83.11% and 80.33% respectively) (Basalingappa, 2011). Azoxystrobin was totally ineffective in inhibiting the mycelial growth of *C. gloeosporioides* from nutmeg which was in contradiction to the observations made by Adhikary *et al*. (2013) who reported a mycelial inhibition of 99.69 per cent at 100 ppm concentration. Ahmed *et al.* (2014) reported the complete inhibition of *C. gloeosporioides* from betel vineeven at 50 ppm of propiconazole, tricyclazole and tebuconazole.

In vitro evaluation of various fungicides carried out by Dev and Narendrappa (2016) revealed that trifloxystrobin + tebuconazole 75 WG (100 ppm, 250 ppm, 500 ppm and 1000 ppm) and triazole fungicide propiconazole (500ppm, 1000 ppm and 2000 ppm) gave 100 per cent mycelial inhibition. Difenoconazole 25 SC (1000 ppm) showed a mycelial inhibition of 85.85 per cent. Strobilurin fungicide, azoxystrobin 25 SC (1000 ppm) was found to show a minimum mycelial inhibition of 52.64 per cent. Parvathy and Girija (2016) reported fungicides *viz*., propiconazole, tebuconazole, azoxystrobin and carbendazim + mancozeb, captan + hexaconazole each at 0.1 per cent completely inhibited the mycelial growth of *C. gloeosporiodies* causing black pepper anthracnose. Behera *et al. (*2019) evaluated the efficacy of carbendazim, mancozeb and its combination fungicide (carbendazim + mancozeb) along with the biocontrol agents in inhibiting the *C. gloeosporioides* from black pepper. Carbendazim + mancozeb @ 0.1 per cent exhibited a maximum inhibition of 97.26 per cent under *in vitro* conditions. The results of the study were similar with respect earlier authors. The results of the present study are in par with Asalkar *et al*. (2019) in which the fungicides carbendazim + mancozeb and propiconazole were found effective with 100% and 97.51% mycelial inhibition respectively. The results of cent percent mycelial inhibition by carbendazim + mancozeb is in agreement with the results obtained by Poonacha *et al.*, (2020). Positive correlation of results is found with results reported by Marak *et al*., (2020). Least mycelial growth in fungicide amended media was recorded by combination fungicides carbendazim + mancozeb 0.1% and trifloxystrobin + tebuconazole 0.1% with 100% inhibition in which the results of coincides with the current study. Among the six fungicides, carbendazim 12% WP + mancozeb 63% WP was found to be effective in inhibiting the mycelial growth of the pathogen even at a lower concentration of 25 ppm. Propiconazole 25 EC was the most effective triazole fungicide against the anthracnose of nutmeg.

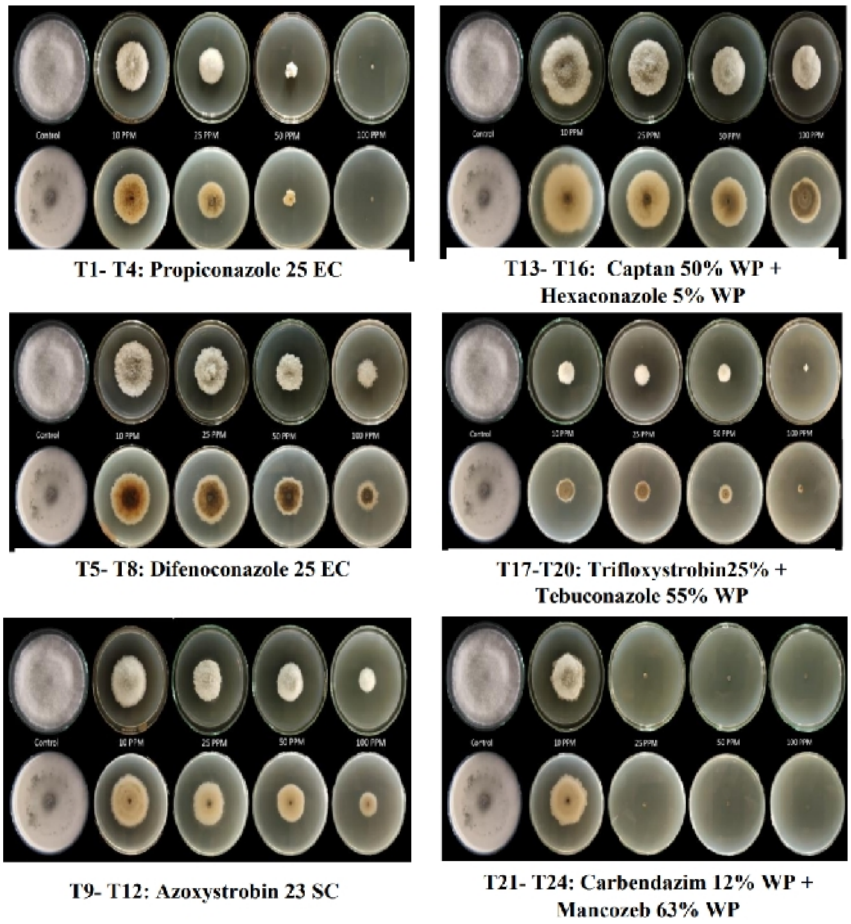

Fig. 2. Nature of mycelial growth *C. gloeosporioide*s (C4) in PDA medium amended with various fungicides at 7 DAI.

CONCLUSION

In the present study, among the six new generation fungicides used, carbendazim 12% WP + Mancozeb63% WP was found to be most effective in inhibiting the growth of the mycelium even 100% even at a low concentration of 25 ppm and 50 ppm. This treatment was found to be significantly superior to all the other fungicides used for the evaluation. Arbendazim 12% WP + Mancozeb 63% WP among combination fungicides and Propiconazole 25 EC among the triazole fungicides were found effective in managing the anthracnose disease of nutmeg. The future line of work should include molecular variability between various isolates, cross infectivity among the isolates in other perennial hosts, and the efficacy of new generation fungicides under field condition.

Acknowledgement. The First author is extremely thankful to the Department of Plant Pathology, Vellayani, Thiruvananthapuram, Kerala Agricultural University, for the successful completion of this research work by providing all the facilities, funding and fellowship.

Conflict of Interest. None.

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How to cite this article: Bommana Divya, Heera G., Joy Michal Johnson, Radhika N.S. and Sreekala G.S. (2022). Efficacy of New Generation and Combination Fungicides under *in vitro* for the Management of Anthracnose of nutmeg (*Myristica fragrans* Houtt.). *Biological Forum – An International Journal*, *14*(2): 1206-1210.